

GRID and docking analyses reveal a molecular basis for flavonoid inhibition of src-family kinase activity

Article

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Title: GRID and Docking Analyses Reveal a Molecular Basis for Flavonoid Inhibition of Src-Family Kinase Activity

Article Type: Research Article

Keywords: Flavonoid molecular templates, GRID, Sybyl docking, selective flavonoid-based analogues, kinase inhibition, flavonoid computational studies, cardiovascular disease and flavonoids, anti-platelet agents and flavonoids

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Abstract: Flavonoids reduce cardiovascular disease risk through anti-inflammatory, anti-coagulant and anti-platelet actions. One key flavonoid inhibitory mechanism is blocking kinase activity that drives these processes. Flavonoids attenuate activities of kinases including phosphoinositide-3-kinase (PI3K), Fyn, Lyn, Src, Syk, PKC, PIM1/2, ERK, JNK, and PKA. X-ray crystallographic analyses of kinase-flavonoid complexes show that flavonoid ring systems and their hydroxyl substitutions are important structural features for their binding to kinases. A clearer understanding of structural interactions of flavonoids with kinases is necessary to allow construction of more potent and selective counterparts.

We examined flavonoid (quercetin, apigenin and catechin) interactions with Src-family kinases (Lyn, Fyn and Hck) applying the Sybyl docking algorithm and GRID. A homology model (Lyn) was used in our analyses to demonstrate that high quality predicted kinase structures are suitable for flavonoid computational studies. Our docking results revealed potential hydrogen bond contacts between flavonoid hydroxyls and kinase catalytic site residues. Identification of plausible contacts indicated that quercetin formed the most energetically stable interactions, apigenin lacked hydroxyl groups necessary for important contacts, and the non-planar structure of catechin could not support predicted hydrogen bonding patterns. GRID analysis using a hydroxyl functional group supported docking results. Based on these findings, we predicted that quercetin would inhibit activities of Src-family kinases with greater potency than apigenin and catechin. We validated this prediction using in vitro kinase assays.

We conclude that our study can be used as a basis to construct virtual flavonoid interaction libraries to guide drug discovery using these compounds as molecular templates.

From

Bernice Wright, Ph.D

b.wright@ucl.ac.uk

02nd May, 2015

Dear Dr Richardson,

Please find attached a copy of the research article entitled “GRID and Docking Analyses Reveal a Molecular Basis for Flavonoid Inhibition of Src-Family Kinase Activity” for consideration for publication in *Journal of Nutritional Biochemistry*. This is original work that has not been published in any form and is not under consideration elsewhere. The manuscript has been read and approved by each author.

The mechanisms of action of flavonoids are of particular interest is their ability to function as protein and lipid kinase inhibitors. We have previously described structure-activity studies that reinforce the possibility for using flavonoid structures as templates for drug design. In the present study we examined flavonoid (quercetin, apigenin and catechin) interactions with Src-family kinases (Lyn, Fyn and Hck) using an *in silico* approach applying the Sybyl docking algorithm and GRID. We performed this study to begin clarifying our understanding of structural interactions of flavonoids with kinases to allow construction of more potent and selective counterparts derived from these compounds. Our study presents methodology that may be used to construct virtual libraries of flavonoid interactions to guide drug discovery using these compounds as molecular templates.

We would be very grateful for this work to be considered for publication in *Journal of Nutritional Biochemistry*. We look forward to hearing from you in due course.

Yours sincerely,

Bernice Wright
Research Fellow

From

Bernice Wright

b.wright@ucl.ac.uk

02nd May, 2015

Dear Dr. Richardson,

Please find attached a copy of the research manuscript entitled “GRID and Docking Analyses Reveal a Molecular Basis for Flavonoid Inhibition of Src-Family Kinase Activity”. We have performed a revision of our manuscript, incorporating all changes suggested by the reviewers. We thank the reviewers for their suggestions to improve the manuscript and they have been addressed as follows:

Reviewer 1

1. In the Highlights, your first point should be revised to summarize what you did, not what could be done.

We apologise for this error and we have corrected the first point in the Highlights: Docking, interaction mapping (GRID), and protein modelling methodology are used to indicate how the study of flavonoid interactions with kinases can guide drug discovery using these compounds as molecular templates.

2. The use of the term "homologous" is not quite correct on page 5, Results/Discussion.

We thank the reviewer for the thoughtful correction of the term ‘homologous’. We have replaced this term with ‘structurally similar’.

3. With regard to Figure 1, you refer to various hydroxyl positions (i.e., C-3) that are not correspondingly numbered in the figure.

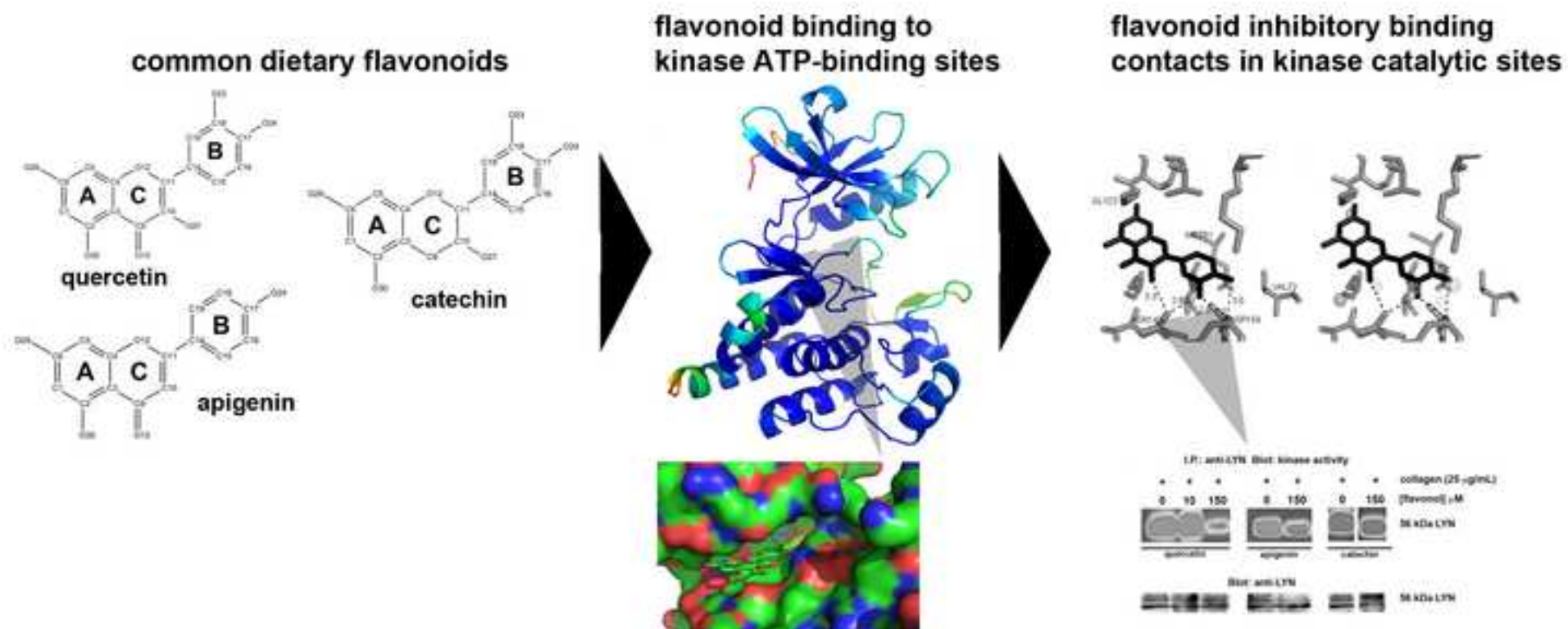
We apologise for this error. We have reviewed the full manuscript and we have changed labels for hydroxyl groups to correspond with the numbering scheme in Figure 1.

We look forward to your reply in due course.

Yours sincerely,

Bernice Wright

Research Fellow



Highlights

- Docking, interaction mapping (GRID), and protein modelling methodology are used to indicate how the study of flavonoid interactions with kinases can guide drug discovery using these compounds as molecular templates.
- High quality predicted kinase structures are suitable for flavonoid computational studies.
- Structural interactions of flavonoids with kinases are necessary to allow construction of more potent and selective counterparts derived from these compounds.

GRID and Docking Analyses Reveal a Molecular Basis for Flavonoid Inhibition of Src-Family Kinase Activity

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Short Title: Molecular Basis for Flavonoid/kinase Interactions

Abstract

Flavonoids reduce cardiovascular disease risk through anti-inflammatory, anti-coagulant and anti-platelet actions. One key flavonoid inhibitory mechanism is blocking kinase activity that drives these processes. Flavonoids attenuate activities of kinases including phosphoinositide-3-kinase (PI3K), Fyn, Lyn, Src, Syk, PKC, PIM1/2, ERK, JNK, and PKA. X-ray crystallographic analyses of kinase-flavonoid complexes show that flavonoid ring systems and their hydroxyl substitutions are important structural features for their binding to kinases. A clearer understanding of structural interactions of flavonoids with kinases is necessary to allow construction of more potent and selective counterparts.

We examined flavonoid (quercetin, apigenin and catechin) interactions with Src-family kinases (Lyn, Fyn and Hck) applying the Sybyl docking algorithm and GRID. A homology model (Lyn) was used in our analyses to demonstrate that high quality predicted kinase structures are suitable for flavonoid computational studies. Our docking results revealed potential hydrogen bond contacts between flavonoid hydroxyls and kinase catalytic site residues. Identification of plausible contacts indicated that quercetin formed the most energetically stable interactions, apigenin lacked hydroxyl groups necessary for important contacts, and the non-planar structure of catechin could not support predicted hydrogen bonding patterns. GRID analysis using a hydroxyl functional group supported docking results. Based on these findings, we predicted that quercetin would inhibit activities of Src-family kinases with greater potency than apigenin and catechin. We validated this prediction using *in vitro* kinase assays.

We conclude that our study can be used as a basis to construct virtual flavonoid interaction libraries to guide drug discovery using these compounds as molecular templates.

Keywords

Flavonoid molecular templates, GRID, Sybyl docking, selective flavonoid-based analogues, kinase inhibition, flavonoid computational studies, cardiovascular disease and flavonoids, anti-platelet agents and flavonoids

1.1. Introduction

Bioactive, plant-derived flavonoids impact on the function of the vascular system through inhibition of the activity of kinases which regulate cell proliferation [1-3], the immune response [4-7], inflammatory processes [5-6], blood coagulation [8] and platelet-mediated thrombosis [9-11]. Flavonoids can achieve these effects by gaining direct access to signalling kinases in the cytosolic compartment [12-16]. A number of reports demonstrate that flavonoids are versatile and effective kinase inhibitors [1-3, 5, 7, 13, 17-32], but they also show that these compounds are not selective. The aim of this study is to apply complementary computational methodologies to elucidate specific and important flavonoid interactions with Src-family kinases. The work demonstrates that homology models can be used for these studies together with predictive computational methodology to generate data that can guide functional studies demonstrating biological validation of predicted flavonoid interactions with kinases. This approach to screening flavonoid activities for kinases will accelerate the process of understanding the molecular interactions of flavonoids with kinases and allow translation of these compounds to more potent and selective analogues. The present study provides a basis for virtual experimental methodologies to explore structural features of these compounds, which confer selectivity and potency toward Src-family kinases.

A number of functionally-diverse kinases (myosin light chain-kinase, PKC and PKA [25]) with a central involvement in the growth, proliferation and functional maintenance of nucleated cells and key regulatory roles in signal transduction in platelets were incorporated into initial studies investigating flavonoids as molecular probes for enzyme/kinase catalytic sites. Previous reports also demonstrated that congeneric flavonoids (quercetin, catechin, apigenin) and their physiological metabolites inhibit the function of platelets by interfering with the activities of tyrosine (Syk, Fyn and Lyn) [13, 18, 30-31] and lipid (PI3K) [18] kinases, as well as phospholipases (phospholipase C γ 2) [13, 18, 30-31]. These compounds exert pro-oxidant and antioxidant effects on the production of reactive nitrogen [32] and oxygen [33-34] species respectively, by targeting the activatory motifs of membrane-bound proteins including the FcR γ chain [18] and Linker and Activator of T cells [18].

Furthermore, studies have shown that flavonoids inhibit the activities of kinases in vascular and immune cells. Red wine polyphenolic compounds containing high levels of flavonoids inhibit the phosphorylation of serine/threonine kinases, p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase and protein kinase B/Akt in vascular smooth muscle cells (VSMC) [1] and endothelial cells [3]. The phytoestrogen, genistein, was recently reported to inhibit high glucose-induced adhesion of monocytes to human aortic endothelial cells by inhibiting adenylate cyclase and protein kinase A (PKA) [5]. The flavone, luteolin was reported to inhibit VSMC proliferation by blocking the activities of Akt and Src [2]. Other flavonoid subgroups including flavanones (hesperidin, naringin) blocked high glucose-induced phosphorylation of p38 MAPK in monocytes [6]. The complex flavan-3-ol, epigallocatechin-3-gallate (EGCG) was suggested to inhibit mast cell-dependent allergic reactions *in vivo* by blocking the activities of tyrosine (Fyn, Lyn, Btk, Syk) and serine/threonine (Akt and c-Jun N-terminal kinase) kinases [7]. EGCG was also shown to reverse the progression of immune-mediated glomerulonephritis, partly by reducing oxidative stress through inhibition of inducible nitric oxide synthase, nitric oxide metabolites, p-Akt, phosphorylated ERK1/2, p47phox, and myeloperoxidase [4].

These studies indicate that flavonoids interact with kinases on a molecular level, and X-ray crystallographic analyses of kinase-flavonoid complexes demonstrate that the flavonoid ring systems and their hydroxyl substitutions determine the specificity of binding of these compounds to Src-family kinases (Hck) [35], lipid kinases (PI3K γ) [27], serine/threonine kinases (PIM1) [24], and DNA gyrase [36]. Molecular docking analyses support such structural studies. Monomeric flavonoids were shown in docking studies to be accommodated in specific binding sites found on Raf1 kinases [28] as well as serine proteases [37] involved in blood coagulation and the inflammatory response.

In the present study, we investigated potential binding modes (docking) and molecular interactions (GRID) of flavonoids (quercetin, apigenin and catechin: **Figure 1.**) with solved (Fyn and Hck) and modelled (Lyn) Src family kinases using GRID and docking algorithms. We suggested inhibitory potencies for these compounds based on docking and GRID data, and we authenticated these potencies using *in vitro* kinase activity assays. We report that computational

analysis approximating flavonoid interactions with modelled and crystallised kinases, together with biological validation, may direct and accelerate virtual screening studies for translation of these compounds into selective and potent small-molecule inhibitors applied to the discovery of therapeutic agents for vascular disorders.

2.1. Results/Discussion

2.1.1. The Human Lyn Kinase Model is Structurally Robust and is Structurally Similar to the Crystal Structure of the Lyn Kinase Domain

We constructed a model of the Lyn kinase domain to validate the use of homology models in our computational approach for investigating flavonoid binding within Src family kinase catalytic sites (**Figure 2.**). A model of the Lyn kinase domain was built based on the Lyn kinase domain from *Mus musculus* (PDB-ID 2h8h) template (**Figure 3A.**) using the ESyPred3D online server. The model (**Figure 3B.**) was structurally similar to the crystal structure of the human Lyn kinase domain (PDB-ID 3a4o) (**Figure 3C.**) - the root mean square deviation (RMSD) was 1.6 Å. The ModFOLD server (<http://www.reading.ac.uk/bioinf/ModFOLD/>) [38-39] was used to evaluate global and local model quality prior to docking.

Our approach is supported by reports [40-44] describing the use of homology models for virtual screening in structure-based drug design. This methodology has been demonstrated to be reliable in accurately predicting the structure of proteins, particularly in those cases where the target is structurally similar to the template. Computer-aided drug design approaches can complement homology modelling approaches to provide a completely virtual environment for drug discovery. A typical structural bioinformatics workflow includes characterization of a protein target, modelling the protein using sequence homology, optimization of the protein structure and finally docking of small ligands into the active site. Previous studies have demonstrated interactions of modelled proteins with small molecules which were subsequently validated using *in vitro* biological assays. The discovery of novel PTP1B inhibitors [40], Cdc25 phosphatase inhibitors [41], and angiogenin inhibitors [42] has been enabled through the use of virtual screening strategies.

For our specific problem involving the use of flavonoids as templates for drug design, high quality kinase homology models will increase the efficiency of screening because they permit the availability of a wide range of kinases. The extensive sequence information that is currently available indicates that there are an increasing number of proteins as potential drug targets with unknown structures [44]. Our approach using protein models as well as solved protein structures strengthens the possibility of screening the entire flavonoid family of compounds against all kinases that they are likely to target *in vivo*.

2.1.2. Flavonoid Docking and GRID Interactions within Lyn, Fyn and Hck ATP Binding Sites Indicate their Potencies for Inhibition of Kinase Activity

Our previous studies demonstrated key structural differences between flavonoids for inhibition of kinase-dependent signalling in platelets [13]. In the present study, we have performed *in silico* evaluations of the binding poses (via docking using the Sybyl algorithm) and functional group interactions (via interaction energy predictions using the GRID algorithm) between quercetin, apigenin and catechin and the catalytic sites of Lyn, Fyn and Hck Src-family kinases, to examine potential underlying molecular interactions for each of these compounds and the kinases of interest. Quercetin, apigenin and catechin were manually docked into the substrate binding groove/ATP-binding site in the hinge region between the N- and C-lobes in Lyn, Fyn and Hck kinases. The Sybyl docking programme was used to predict potential interactions of the flavonoids with residues in the kinase ATP binding sites, and the GRID programme was run to predict corresponding interaction energies of hydrophilic and hydrophobic regions. The GRID programme [45] predicted three dimensional energy contour surfaces/regions (energy minima were displayed as dotted regions) within the substrate binding grooves of Hck, Fyn (crystal structures) and Lyn (homology model) corresponding to the energy minima of hydrophilic (OH) and hydrophobic (DRY) small chemical probes representative of flavonoid functional groups (OH - hydroxyl groups; DRY - aromatic rings). Used in this manner, the GRID programme was applied to independently verify the docking results. The Sybyl programme utilised a semi-flexible docking algorithm to dock the flavonoids into the ATP-binding site of the selected Src family kinases. The performance of the Sybyl algorithm [46] in ligand docking and scoring was

judged by its ability to reliably reproduce interactions observed for a known inhibitor-kinase complex (quercetin-Hck complex, PDB-ID 2HCK), by way of a positive control.

We extracted the X-ray pose of quercetin from within the ATP binding site of Hck (PDB-ID 2HCK - reference structure), for use as a control. Additionally, a model of quercetin was built, minimised and docked into the crystal structure of Hck. The docked pose for quercetin, from the model, superimposed onto the reference model of quercetin taken from the co-crystallised complex (**Figure 4B.**) with an RMSD of 1.372 Å, which is well within the generally accepted values of 1.5 to 2 Å. A cluster of hydrophilic interactions were observed adjacent to interactions between ASN141 and ASP154 the C ring and B ring hydroxyls in the Lyn kinase domain (**Figure 4B.**). Modelled quercetin docked into Hck with the chromone moiety (A-C ring complex) directly adjacent to glycine (GLY344) and methionine (MET341) (**Supplemental Figure 2A.**). These data were supported by GRID, which showed areas of hydrophilic interactions (GLY344 and the C ring C-10 hydroxyl) corresponding to the hydrogen bonds revealed by docking (**Supplemental Figure 2B.**). Quercetin co-crystallised with Hck [35] also formed hydrogen bonds with methionine residues (MET341). Within the ATP-binding site of Fyn (**Supplemental Figure 1A. and 1B.**), quercetin docked in a similar orientation as Hck; hydrogen bonds were formed between the chromone moiety and methionine (MET835) and between ASP154 and LYS39. Hydrophilic GRID interactions adjacent to MET85 were observed, and the hydrogen bond contacts formed between ASP148 and LYS39 and B ring hydroxyls were surrounded by hydrophilic GRID regions. Our published work has shown that quercetin inhibits Fyn kinase activity with high potency; the predicted pose for the flavonol, which optimises the interactions with MET85 and GLY88 may account for its potency [13]. Therefore, the predicted pose of quercetin bound to Fyn, where the chromone moiety is involved in hydrogen bond interactions, may be correlated to high potency inhibition. Within the Lyn homology model, this flavonoid docked in a similar orientation as that found in Fyn and Hck; towards asparagine and glycine residues (Lyn: ASN141, GLY23) (**Figure 5A. and 5B.**). Previous studies with fisetin [47] (bound to the active form of CDK6), have also shown that quercetin forms hydrogen bonds with the side chains of residues in the binding pocket. This binding causes large conformational changes during CDK activation by cyclin binding. The 4-keto group and the 3-hydroxyl group of fisetin are hydrogen bonded with the backbone in the hinge region between the N-terminal and C-terminal kinase domain, as has been observed for many CDK inhibitors. However, CDK2 and

HCK kinase in complex with other flavone inhibitors, e.g. flavopiridol, showed a different binding mode with the inhibitor rotated by about 180 degrees.

Apigenin docked within Lyn (**Figure 5C. and 5D.**), Hck (**Supplemental Figure 1C. and 1D.**) and Fyn (**Supplemental Figure 2C. and 2D.**) with a similar number of hydrogen bonds as quercetin, suggesting that these flavonoids may inhibit Src-family kinase activity with similar potency. However, apigenin has been reported previously as a poor inhibitor of Fyn kinase activity, whilst quercetin was shown to be a high potency inhibitor [13]. It is possible that the hydrogen bond formed between the methionine (MET91: Lyn and MET341: Hck) and the para-hydroxyl in the A ring of apigenin (observed in Lyn and Hck ATP docking, but not Fyn) is necessary. GRID interactions supported the hydrogen bonds that were formed. In the Lyn kinase domain MET91 and GLU89 hydrogen bonds with the A ring C-6 hydroxyl are adjacent to favourable hydrophilic GRID regions. ASN19 binding to the A ring C-6 hydroxyl in Fyn as well as the THR82 and GLU83 bound to the B ring C-17 hydroxyl are also near favourable hydrophilic GRID regions. Within the Hck kinase domain, MET341 binds to the A ring C-6 hydroxyl and SER345 and ASP348 bind to the B ring C-17 hydroxyl. Quercetin does not directly interact with methionine (MET91) in the Lyn kinase domain and, as the flavonol is a potent inhibitor of Lyn, the C ring C-10 hydroxyl must also be important. Therefore, apigenin may be less potent than quercetin due to lower affinity caused by omission of the C ring C-10 hydroxyl from the structure of the flavone. Apigenin may be more selective for Src than for Lyn, Fyn and Hck, as previous studies demonstrate; in *in vitro* pull-down assays the flavone binds Src in an adenosine triphosphate-competitive manner [48].

Within the ATP binding pocket of Lyn (**Figure 5E. and 5F.**), catechin docked in an opposite orientation to that of quercetin, but a similar orientation to apigenin. The B-ring of the flavan-3-ol was positioned adjacent to the methionine residue (MET91). Similar to quercetin, the C ring C-10 hydroxyl oxygen formed a hydrogen bond with the methionine (MET341) residue, in Hck (**Supplemental Figure 1E. and 1F.**) and Fyn (MET85) (**Supplemental Figure 2A. and 2B.**). Catechin also docked with a similar number of hydrogen bonds as quercetin, in Fyn, Lyn and Hck binding sites. These data suggest that the orientation of the flavonoids within kinase ATP-binding sites rather than the number of hydrogen bonds formed may determine their inhibitory potency. Although these data suggested that catechin may inhibit Src-family kinase activity with similar high potency as quercetin, previous reports have demonstrated that this

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4 compound inhibits Fyn kinase activity with low potency. GRID hydrophilic interactions
5
6 corresponded to hydrogen bonds formed between GLU89, MET91 and ALA92 and A and B ring
7
8 hydroxyl oxygens in the Lyn kinase domain, those formed between MET85 and C ring C-10
9
10 hydroxyls in the Fyn kinase domain, and those between MET341, ALA342 and LEU273 and C
11
12 and A ring hydroxyls. Therefore, although the hydroxyl groups on the non-planar C-ring form
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14 hydrogen bonds, these may not be energetically favourable. Previous studies have shown that
15
16 within the ATP-binding site of DNA gyrase, epigallocatechin gallate (EGCG) a structural
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18 homologue of catechin (epicatechin), was orientated in a manner opposite to that of quercetin and
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20 a network of hydrogen bonds was formed between the flavonol and neighbouring residues but
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22 hydrogen bonds only formed between residues and the B ring of the epicatechin moiety [49].
23
24 Moreover, specific knockdown of Fyn (but not Src) with small interfering RNA inhibited both
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26 EGCG-stimulated phosphorylation of Akt and endothelial nitric oxide synthase as well as
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28 production of nitric oxide in bovine aortic endothelial cells [50]. Furthermore, in an *in vitro*
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30 protein-binding assay, EGCG was found to directly bind with the GST-Fyn-SH2 domain but not
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32 the GST-Fyn-SH3 domain [51]. Therefore, complex flavan-3-ols targeted at Src homology
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34 domains may prove to be a more viable means of achieving potent inhibition of Src-family
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36 kinases than catechin.

37
38 The docking results suggest that catechin may be only partially anchored within Src-
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40 family kinase binding pockets via A-ring hydroxyl bonds, whereas quercetin and apigenin
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42 molecules with planar C rings are potentially fully anchored within these kinases. The fact that
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44 catechin is only partially anchored in the binding pocket also suggests that solvation effects may
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46 be playing a role here. The planarity of the flavonoid chromone moiety has been reported to be
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48 essential for the inhibitory activity of these compounds [13]. Therefore, quercetin and apigenin
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50 may be more potent inhibitors of Src-family kinase activity than catechin, due to more stable
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52 binding.
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2.1.3. Flavonoid Inhibitory Potencies for Lyn and Hck Suggested from Docked Conformations and GRID Interactions are Validated by *in vitro* Kinase Activity Assays

Quercetin inhibited the activities of Lyn and Hck to a significantly greater extent than both apigenin and catechin (**Figure 6.**). Therefore inhibitory potencies, from approximated binding conformations (docking) and functional group interactions (GRID) suggesting that quercetin was a more potent inhibitor than apigenin and catechin, were substantiated. Quercetin (150 μM) inhibited Hck kinase activity 9-fold greater than apigenin (150 μM) but only 3-fold greater than catechin (150 μM) (**Figure 6B. and 6C.**). Quercetin also exerted inhibition of Hck kinase activity at 10 μM , a concentration that was achieved *in vivo* in human subjects following ingestion of a quercetin glucoside supplement. These differences indicated that C-ring hydroxylation (apigenin) may be more important than C-ring planarity (catechin) for potent inhibition of Src-family kinase activity, and the non-planar C ring C-10 hydroxyl (catechin) may elicit an inhibitory effect. Inhibition of Lyn kinase activity by quercetin was 4-fold greater than that mediated by both apigenin and catechin (**Figure 6A. and 6C.**). The differences in inhibition may be due to binding to antibodies [52] as Lyn kinase was immunoprecipitated, whereas Hck was a recombinant protein.

Recent studies show that flavonoids can bind directly to protein kinases, including Akt/protein kinase B (Akt/PKB), Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1 (MEK1), phosphoinositide 3-kinase (PI3K), mitogen-activated protein (MAP) kinase kinase 4 (MKK4), Raf1, and zeta chain-associated 70-kDa protein (ZAP-70) kinase, and alter their phosphorylation state to regulate multiple cell signalling pathways [53]. Apigenin, luteolin and quercetin that have been reported to inhibit GSK-3 β [54], with 50% inhibitory values of 1.5, 1.9, and 2.0 μM , respectively, were predicted to fit within the binding pocket of GSK-3 β with low interaction energies (-76.4, -76.1, and -84.6 kcal $\cdot\text{mol}^{-1}$), respectively) and low complex energies (-718.1, -688.1, and -719.7 kcal $\cdot\text{mol}^{-1}$), respectively).

Previous studies have described results that are in line with those presented in the current study, as they demonstrate that flavonoid attenuation of, and binding to lipid [23, 36] and serine/threonine kinases [22-24] is dependent on a C ring C-10 hydroxyl for high potency inhibition. Substitution of the C ring C-10 hydroxyl has been shown to reduce the IC₅₀ of quercetin analogues [55]. By contrast, substitution of the C ring C-10 hydroxyl with an amine

group was shown to confer greater selectivity for Src than the epidermal growth factor receptor [56]. Removal of the C ring C-10 and B ring C-16 hydroxyls (apigenin) and addition of a C-17 methyl group to the B ring of quercetin (tamarixetin) correlated with low potency inhibition of Fyn involved in GPVI signalling [13]. Hydroxylation of the A ring may also be important for binding because quercetagenin (with an A ring C-1 hydroxyl that is omitted from the structure of quercetin) was demonstrated to be more potent than quercetin in the ATP binding sites of *pim* kinases [24]. Modification of A and B rings with phenol group (LY294002: analogue derivative of quercetin) on an unmodified chromone moiety also increases potency and selectivity of quercetin [57].

Previous docking studies of flavonoid-kinase inhibition substantiated by biological assays have also indicated that these compounds may be selective. A report described that Fyn kinase, not Lyn and Syk, was inhibited by morin in a dose-dependent manner (IC₅₀: 5.7 μM). Kaempferol-7,4'-dimethylether was previously demonstrated as a potent p38α inhibitor, displaying 13-fold selectivity for p38α over JNK3 [58]. Flavone compounds without a 6-methoxy group preferentially inhibited JNK3; luteolin-7-O-glycoside, was identified as a potent inhibitor with the greatest selectivity toward JNK3. Flavanol compounds, however, were shown to display similar inhibitory activities toward both kinases. Delphinidin strongly inhibited TNF-α-induced COX-2 expression in JB6 P+ mouse epidermal (JB6 P+) cells, whereas two other major phenolic compounds (resveratrol and gallic acid) did not exert significant inhibitory effects [59]. Delphinidin inhibited the TNF-α-induced phosphorylations of JNK, p38 MAP kinase, Akt, p90RSK, MSK1, and ERK, and subsequently blocked the activation of the eukaryotic transcription factors AP-1 and NF-κB. Kinase and pull-down assay data revealed that delphinidin inhibited Fyn kinase activity and directly bound with Fyn kinase noncompetitively with ATP [59]. This may enable us to perform pharmacophore studies with our work as a basis. A pharmacophore model for ATP-competitive inhibitors interacting with the active site of the EGFR protein tyrosine kinase, together with published X-ray crystal data of quercetin in complex with the Hck tyrosine kinase, and deschloroflavopiridol in complex with CDK2, a putative binding mode of the isoflavone genistein was previously proposed [60]. Based on literature data suggesting that a salicylic acid function, i.e. the 5-hydroxy-4-keto motif in genistein, could serve as a pharmacophore replacement of a pyrimidine ring, superposition of genistein onto the potent EGFR tyrosine kinase inhibitor 4-(3'-chlorophenylamino)-6, 7-dimethoxyquinazoline led to the

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4 formation of 3'-chloro-5,7-dihydroxyisoflavone. This target structure was 10 times more potent
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6 than genistein.
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10 11 **3.1. Conclusions**

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13 We conclude that both computational and experimental methodologies may be used together to
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15 understand flavonoid-kinase molecular interactions. Dissections of interactions between key
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17 flavonoid functional groups and kinases have revealed important information about structural
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19 features underlying the inhibitory potencies of these compounds. Derivation of potent, selective
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21 small-molecule inhibitors from flavonoids is therefore possible. Flavonoid analogues have been
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23 constructed, but a clear understanding of the manner in which functional groups on these
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25 compounds associate with molecular targets is crucially important for substantial progress to be
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27 made in this venture.

28 We describe the basis for an organised experimental strategy based on computational
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30 guidelines that may begin to form an interaction library and help direct further design for rational
31
32 screening of the flavonoid structure. Our application of complementary computational algorithms
33
34 considers the inclusion of kinases with both solved structures (X-ray or NMR) and homology
35
36 models to investigate the molecular interactions of key functional groups on the flavonoid
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38 structure (using GRID and docking programmes) within the Src-family kinase ATP binding site.
39
40 Our method is an important pre-requisite to structure-function work that will ensure efficient
41
42 dissection of the complete family of compounds within the flavonoid polyphenolic subgroup.

43 The findings presented here form the basis for further combined computational and
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45 structural studies to fully elucidate and exploit the key molecular interactions between Src-family
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47 kinases and flavonoids. With rigorous validation of computational modelling approaches, all
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49 plausible permutations of flavonoid interactions with signalling kinases of target cells may be
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51 explored and catalogued for application to drug design.
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4.1. Experimental Section

4.1.1. Ethics Statement

Blood was obtained from healthy aspirin-free human volunteers with written informed consent, following approval from the University of Reading Research Ethics Committee.

4.1.2. Materials

Quercetin, apigenin and catechin were purchased from Extrasynthese (Genay, France). Flavonoids were solubilised in dimethylsulphoxide (DMSO) obtained from Sigma (Poole, UK). Protein A Sepharose (PAS: from *Staphylococcus aureus*) and silver nitrate were also purchased from Sigma. Collagen (Type I (fibrillar) from equine tendons) was from Nycomed (Munich, Germany) and the anti-Lyn and anti-Hck primary antibodies were obtained from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd; Calne, Wilts, UK). Recombinant Hck protein was obtained from New England Biolabs (Hitchin, Herts, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from GE Healthcare (Little Chalfont, UK). The chemiluminescence detection system was obtained from Pierce (Thermo Fisher Scientific; Rockford, IL USA). The GRID programme suite was licensed from Molecular Discovery (Perugia, Italy), the Sybyl programme was licensed from Tripos (St. Louis, USA) and the PyMOL programme was licensed from DeLano Scientific (CA, USA).

4.1.3. Preparation and Stimulation of Platelets

Blood was obtained from healthy, aspirin-free, human volunteers with informed consent, following approval from the University of Reading Research Ethics Committee. Platelets were isolated by differential centrifugation and suspended in modified Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 1 mM MgCl₂, 5 mM glucose, pH 7.3) to a density of 8×10^8 cells·mL⁻¹ in modified Tyrode's-HEPES buffer containing 1 mM EGTA to prevent aggregation. Platelets (450 µL) were incubated with flavonoids or DMSO (1 µL: 0.2% (v/v)) for 5 min (after 10 s stirring) prior to stimulation with

collagen (25 $\mu\text{g.mL}^{-1}$: 50 mL) for 90 s in an optical aggregometer at 37°C with continuous stirring.

4.1.4. Immunoprecipitation and *in vitro* Kinase Assays

Lyn was immunoprecipitated as described previously [18]. Lyn immunoprecipitate and recombinant Hck were suspended in kinase buffer (105 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM MnCl_2 , 5 mM MgCl_2 , 2 mM NaF, 1 mM Na_3VO_4 , 10 μM adenosine triphosphate (ATP)) containing 5 μCi ^{32}P -ATP per reaction, were incubated at 30°C for 20 min with flavonoids or DMSO (0.2% (v/v)) and the kinase reaction terminated through addition of an equal volume of Laemmli reducing sample treatment buffer. Proteins were separated by SDS-PAGE, and transferred to PVDF membranes which were exposed to storage phosphor screens to detect incorporation of ^{32}P (autophosphorylation) into the immobilised kinase.

4.1.5. Immunoblotting

Non-specific binding to PVDF membranes containing proteins, was blocked by incubation with 5% (w/v) bovine serum albumin (BSA) dissolved in 1X Tris-buffered saline-Tween (TBS-T) (20 mM Tris-base, 0.14 M NaCl, 0.1% Tween[®]-20; pH 7.6). Membranes were incubated with primary antibodies (1 $\mu\text{g.mL}^{-1}$) diluted in 2% (w/v) BSA dissolved in 1X TBS-T at 4 °C overnight. Total levels of Lyn and Hck were measured by immunoblotting using anti-Lyn antibody or by silver staining (Hck). Normalisation for protein loading was performed by expressing levels of activity of Lyn and Hck, relative to total levels of those proteins, and was expressed as a percentage compared to the untreated control that represented 100% activity. For immunoblotting, blots were washed for 45 min in 1X TBS-T before incubation with a HRP-conjugated secondary antibody (1:4000 dilution) for 2 h at room temperature (RT) with rotation. Proteins were detected on X-ray film using the chemiluminescence system. Densitometry analysis was performed using ImageQuant software (GE Healthcare: Little Chalfont, UK) and a

BioRad GS710 densitometer with Quantity One analysis software (BioRad; Hemel Hempstead, UK).

4.1.6. Structural Alignments

The Dali-lite structural sieving server was used to perform structural alignments of Lyn, Fyn and Hck kinase domains. This server generates structural alignments by an iterative process involving filtering out residue-residue correspondences until optimal (superimposable) corresponding residues are achieved in structures, under a threshold of RMSD. The lsqkab programme in the CCP4 programme suite was used to perform structural alignments of the crystallised and docked quercetin molecules.

4.1.7. Homology Modelling

Homology models were built for Lyn tyrosine kinase (Homo sapiens) from the Src kinase crystal structure template (PDB ID: 2H8H) using ESyPred3D server [61]. The per-residue error was predicted using the ModFOLD server [38, 62-63]. The colour-coding used by ModFOLD represents the residue accuracy according to a pseudo-temperature scheme (blue indicates residues closest to the native structure; red, those furthest from the native structure). Residues coloured in red are also predicted to have a higher propensity for flexibility according to the DISOclust method [64]. Images were rendered using PyMOL (<http://www.pymol.org>).

4.1.8. GRID Analysis

The GRID programme [45] was utilised to predict potential binding sites, represented as three dimensional energy contour surfaces (or regions), for functional groups within the ATP binding pocket of Hck, Fyn (crystal structures) and Lyn (homology model) kinase domains. The resulting energy contours indicate the location of energetically favourable interactions, with negative

energy levels indicating favourable regions. The potential energy (E_{xyz}) between the target (Hck, Fyn or Lyn) and a small chemical probe (OH, OH₂ and DRY) at each node of the GRID was calculated as: $E_{xyz} = \Sigma E_{EL} + \Sigma E_{HB} + \Sigma E_{LJ}$ [47, 66]. ΣE_{EL} is defined as the appropriately modified electrostatic energy, ΣE_{HB} is the hydrogen bonding energy and ΣE_{LJ} is the Lennard-Jones potential energy between probe and target atoms. Successive probe positions were sampled in the same way until each grid point was assigned an energy value. The program GRIN, the first step in the GRID calculation, was used to prepare each of the coordinate files by removing hydrogens (in the case of the Lyn homology model) adding counterions (Na^+ was used in each case) to neutralise the overall charge on the protein taking care not to include counterions near any potential binding sites and including the associated energy variables to individual atom types as defined in GRIN. The move directive in GRID was set to -1 to allow the counterions to move in response to the probe. The GRID calculations were performed using a grid spacing of 0.5 Å in a GRID box defined as a three dimensional grid of points around and within the ATP binding pocket of Hck, Fyn or Lyn (approximately X x Y x Z). Hydrophilic (OH) and hydrophobic (DRY) probes were selected as being most representative of flavonoid functional groups (OH - hydroxyl groups; DRY - aromatic rings), although, additional chemical probes used included O, OFU, hydrogen donor: OH₂, OH, hydrophobic: DRY and hydrogen acceptor: N1+, N1, N:=, O- (default) probes. The program MINIM was used convert the GRID output to a readable format suitable for input to PyMOL (DeLano Scientific) for graphical viewing and interpretation.

4.1.9. Docking Analysis

The crystal structures of Fyn (2DQ7) and Hck (2HCK) were obtained from the protein data bank (<http://www.ebi.ac.uk>). In order to prepare the protein structures for the docking experiments duplicate chains, where present, as well as all water molecules were removed. Additionally, all ligands were extracted from the coordinate files. Finally, the protein structures were prepared for the docking runs using the Biopolymer Structure Preparation Tool implemented in the programme SYBYL. Docking experiments utilized the Surflex-Dock automatic docking algorithm. The structures of quercetin, apigenin and catechin were built using the BUILDER module in InsightII and a conformational minimisation was performed for these ligands using the

consistent valence force field (CVFF) to the lowest energy conformers for docking analyses. Docking was performed by placing the molecule in the binding pocket of the protein (together in one pdb file) before proceeding with the docking algorithm. In order to calculate the hydrogen bond (H-bond), van der Waals (vdW) and hydrophobic interactions, the resulting PDB files from the docking runs were input to the program CONTACTS, available in the CCP4 programme suite. Potential H-bonds were assigned if the distance between two electronegative atoms was less than 3.3 Å, whereas any separation greater than 3.3 Å, but less than 4.0 Å, was considered a vdW interaction.

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Figure Legend

Figure 1. **Flavonoid structures.**

The flavonoid structure comprises an oxygenated heterocyclic middle ring (C ring) flanked by 2 aromatic rings (A and B rings). Flavones (apigenin) are characterised by a non-hydroxylated C ring, whereas flavonol (quercetin) C rings contain a C-10 hydroxyl group. Flavan-3-ols (catechin) are defined by a non-planar, C-10 hydroxylated C ring that is not substituted with a C-9 carbonyl group.

Figure 2. **Structural alignments of Lyn, Fyn and Hck kinase domains.**

The Dali-lite structural sieving server was used to perform structural alignments of Hck (2HCK), Lyn (3A4O) and Fyn (2DQ7) kinase domains. The RMSD for Hck and Lyn was 2 Å, for Hck and Fyn was 1.9 Å, and for Lyn and Fyn was 1.2 Å.

Figure 3. **Homology models built for Lyn tyrosine kinase (Homo sapiens).**

Homology model of human Lyn kinase, based on the 2H8H template, using the ESyPred3D server [61] (A). The per-residue error was predicted using the ModFOLD server [40, 63-64]. The colours represent the residue accuracy according to a pseudo-temperature scheme (blue indicates residues closest to the native structure; red, those furthest from the native structure). Superimposition (performed using Mustang-MR) of the homology model of Lyn kinase domain (blue), from the 2H8H template, and the X-ray crystal structure of human Lyn kinase domain (green, PDB-ID 3A4O) (B). The RMSD between the kinase domains of the homology model and the crystal structure using Dalilite was 1.6 Å. Superimposition of the active site of Lyn kinase domain homology model (blue) and Lyn kinase domain crystal structure (green) (C). Active site residues are highlighted as ball and stick. Reference [61] refers to the use of the methods developed by the authors of the cited publication.

Figure 4. **The active site of Hck kinase domain (PDB-ID 2HCK) containing co-crystallised and docked quercetin molecules.**

An electrostatic surface representation of the homology model of the Lyn kinase domain showing docked quercetin (A) and Hck kinase domain (2HCK) containing co-crystallised (dark blue) and docked (light blue) quercetin (B).). The RMSD between docked and crystallised quercetin molecules was 1.6 Å.

Figure 5. Molecular docking and interaction energies of flavonoids in the substrate binding groove of the modelled Lyn kinase domain.

The docked ligands (quercetin: A, B; apigenin: C, D; catechin: E, F) are shown in bold outline with neighbouring amino acid residues indicated. Hydrogen bonds (depicted by dashed lines) are shown between the ligand and active site residues with the bond lengths given in Angstroms (Å). The GRID programme predicted three dimensional energy contour surfaces/regions (dotted areas around docked ligands – quercetin: B; apigenin: D; catechin: F) within the ATP binding groove of the Lyn model corresponding to the energy minima of hydrophilic (OH: grey dotted areas) and hydrophobic (DRY: black dotted areas), which act as small chemical probes representative of flavonoid functional groups (OH: hydroxyl groups and DRY: aromatic groups). GRID showed areas of hydrophilic interactions (GLY344 and the C ring C-10 hydroxyl) corresponding to the hydrogen bonds formed by quercetin. The A ring C-6 hydroxyl on the structure of apigenin, forming hydrogen bonds with MET91 and GLU89, was adjacent to hydrophilic GRID regions. GRID hydrophilic interactions correspond to hydrogen bonds formed between GLU89, MET91 and ALA92, and the A and B ring hydroxyl oxygens on the structure of catechin.

Figure 6. Flavonoids block the kinase activity of Hck and Lyn

Washed human platelets (8×10^8 cells.mL⁻¹) in the presence of EGTA (1 mM) were stimulated with collagen (25 µg.mL⁻¹) for 90 s. Platelets were lysed with ice-cold 1% NP40 and Lyn was immunoprecipitated. Lyn immunoprecipitates and recombinant Hck were pre-treated with flavonoids (quercetin, catechin or apigenin), or solvent control (DMSO (0.2% v/v)) for 5 min. Immunoprecipitates were assayed for kinase activity (see Materials and Methods). Equivalent protein loading was verified by reprobing for Lyn and silver staining Hck protein. % inhibition of kinase activity is a percentage of the DMSO-treated, collagen-stimulated control (0% inhibition). The bars represent the mean (n = 3) % inhibition of kinase activity for each treatment (± S.E.M).

* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ compared to the control (DMSO-treated, collagen-stimulated platelets).

Supplemental Figure 1. Molecular docking and GRID interaction energies of flavonoids in the ATP binding site of the Hck kinase domain.

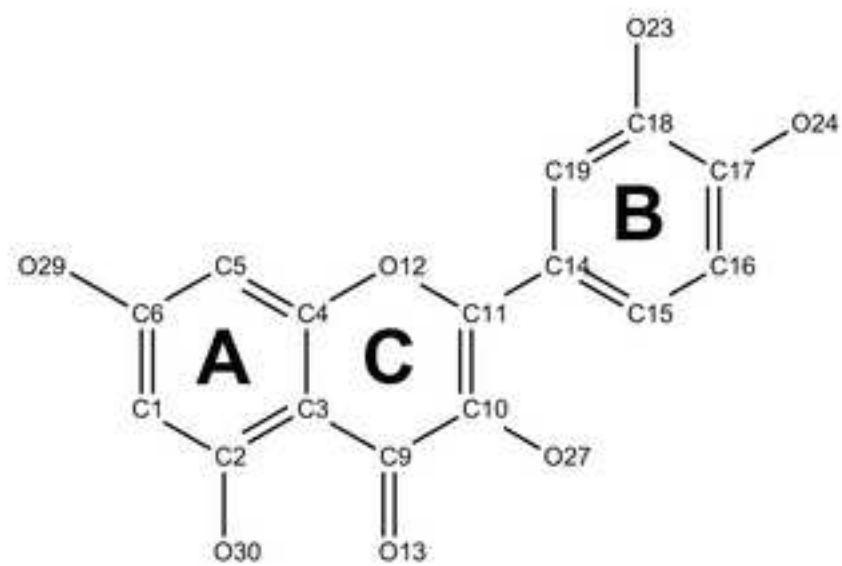
The docked ligands (quercetin: A, B; apigenin: C, D; catechin: E, F) are shown in bold outline with neighbouring amino acid residues indicated. Hydrogen bonds (depicted by dashed lines) are shown between the ligand and binding site residues with the bond lengths given in Angstroms (Å). The GRID programme predicted three dimensional energy contour surfaces/regions (dotted areas around docked ligands – quercetin: B; apigenin: D; catechin: F) within the substrate binding groove of the Hck kinase domain (crystal structure) corresponding to the energy minima of hydrophilic (OH: grey dotted areas) and hydrophobic (DRY: black dotted areas) small chemical probes representative of flavonoid functional groups (OH: hydroxyl groups and DRY: aromatic groups). MET85 as well as the hydrogen bond contacts formed between ASP148 and LYS39 and quercetin B ring hydroxyls were surrounded by hydrophilic GRID regions. Apigenin hydrogen bonds (A ring C-6 hydroxyl) to ASN19 as well as the THR82 and GLU83 bound to the apigenin B ring C-4' hydroxyl are near hydrophilic GRID areas. MET85 and catechin C ring C-10 hydroxyls are also adjacent to GRID hydrophilic areas.

Supplemental Figure 2. Molecular docking and GRID interaction energies of flavonoids in the ATP binding site of the Fyn kinase domain.

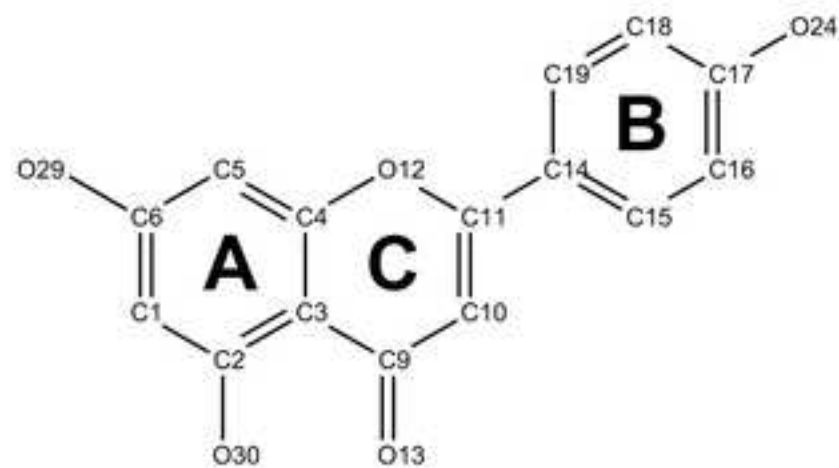
The docked ligands (quercetin: A, B; apigenin: C, D; catechin: E, F) are shown in bold outline with neighbouring amino acid residues numbered. Hydrogen bonds (depicted by dashed lines) are shown between the ligand and binding site residues with the bond lengths given in Angstroms (Å). The GRID programme predicted three dimensional energy contour surfaces/regions (dotted areas around docked ligands – quercetin: B; apigenin: D; catechin: F) within the substrate binding groove of the Fyn kinase domain (crystal structure) corresponding to the energy minima of hydrophilic (OH: grey dotted areas) and hydrophobic (DRY: black dotted areas) small chemical probes representative of flavonoid functional groups (OH: hydroxyl groups and DRY: aromatic

groups). GRID areas of hydrophilic interactions were between the quercetin C ring C-10 hydroxyl and GLY344. MET341 binding to A ring C-6 hydroxyl and SER345 and ASP348 hydrogen bonds to the B ring C-17 hydroxyl on the structure of apigenin are also adjacent to hydrophilic interactions. MET341, ALA342 and LEU273 and C and A ring hydroxyls on the structure of catechin are near hydrophilic GRID regions.

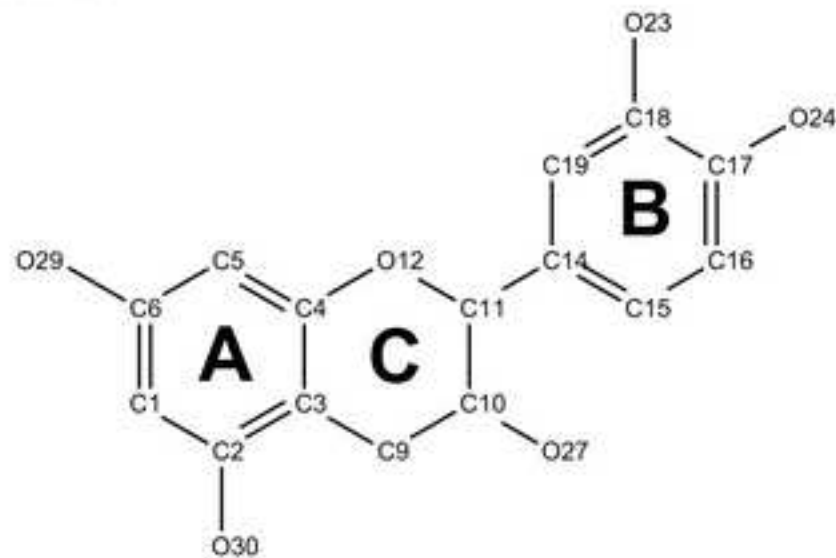
Figure 1
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quercetin



apigenin



catechin

Figure 2
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A	Hck	KDAWEIPRESLKLEKLGAGQFGGEVWQATYNKHTKVAVKTKPGSMSEAFLEAEANVMKT	60
	Lyn	.DAWEIPRESIKLVKRLGAGQFGGEVWQGYNNSTKVAVKTLKPGTMSVQAFLEAEANLMKT	59
	Hck	LQHDKLVLKHAVVTK.EPIYIIITEFMANGSLLDFLKSDGGSKQPLPKLIDFSAQIAEGMA	119
	Lyn	LQHDKLVLRLYAVVTRQEPYIIITEFMANGSLLDFLKSDGGKVLPLPKLIDFSAQIAEGMA	119
	Hck	FIEQRNYIHRLRAANILVSASLVCKIADFGL.arvgakFFIKNTAPEAINFGSFTIKSD	178
	Lyn	YIERKNYIHRLRAANVLVSESLMCKIADFGLarvegakFFIKNTAPEAINFGCFTIKSD	179
	Hck	VNSFGILLMEIVTYGRIPYPGMSNPEVIRALERGYRMPRPENCPEELYNIMRCKNRPE	238
	Lyn	VNSFGILLYEIVTYGKIPYPGRTNADVMTALSQGYRMPRVENCPEELYDINCKCKEKA	239
	Hck	ERPTFEYIQSVLDD.FYTatesq	260
	Lyn	ERPTFDYLQSVLDDFYTAT...e	259
B	Hck	KDAWEIPRESLKLEKLGAGQFGGEVWQATYNKHTKVAVKTKPGSMSEAFLEAEANVMKT	60
	Fyn	KDVWEIPRESQLIKRLGNGQFGGEVWQGTWNGNTKVAIKTLKPGTMSPESELEAQIMNK	60
	Hck	LQHDKLVLKHAVVTK.EPIYIIITEFMANGSLLDFLKSDGGSKQPLPKLIDFSAQIAEGMAF	120
	Fyn	LQHDKLVLQLYAVVSEEPYIIVTEHNGKSLDFLKSDGGKALKLNLVDMQAQVAAGMAY	120
	Hck	IEQRNYIHRLRAANILVSASLVCKIADFGL.....arvgAKFFIKNTAPEAINFG	171
	Fyn	IERNYIHRLRSANILVGNGLICKIADFGLarLiednetarqgAKFFIKNTAPEAALYQ	180
	Hck	SFTIKSDVNSFGILLMEIVTYGRIPYPGMSNPEVIRALERGYRMPRPENCPEELYNIMR	231
	Fyn	RFTIKSDVNSFGILLTELVTGRVPYPGSNREVLEQVERGYRMPCPQDCPISLHELMH	240
	Hck	CKNRPEERPTFEYIQSVLDDFYtatesq	260
	Fyn	CKNKDPEERPTFEYLQSFLEDY.....	262
C	Lyn	.DAWEIPRESIKLVKRLGAGQFGGEVWQGYNNSTKVAVKTLKPGTMSVQAFLEAEANLMKT	59
	Fyn	KDVWEIPRESQLIKRLGNGQFGGEVWQGTWNGNTKVAIKTLKPGTMSPESELEAQIMNK	60
	Lyn	LQHDKLVLRLYAVVTRQEPYIIITEFMANGSLLDFLKSDGGKVLPLPKLIDFSAQIAEGMA	119
	Fyn	LQHDKLVLQLYAVVSE.EPIYIIVTEFMANGSLLDFLKSDGGKALKLNLVDMQAQVAAGMA	119
	Lyn	YIERKNYIHRLRAANVLVSESLMCKIADFGLARV.....EGAKFFIKNTAPEAIN	170
	Fyn	YIERQNYIHRLRSANILVGNGLICKIADFGLARLiednetarqgAKF.FIKNTAPEAAL	178
	Lyn	FGCFTIKSDVNSFGILLYEIVTYGKIPYPGRTNADVMTALSQGYRMPRVENCPEELYDID	230
	Fyn	YGRFTIKSDVNSFGILLTELVTGRVPYPGSNREVLEQVERGYRMPCPQDCPISLHELM	238
	Lyn	NCKCKEKAERPTFDYLQSVLDDFYtate	259
	Fyn	INCKKDPEERPTFEYLQSFLEDY.....	262

Figure 3
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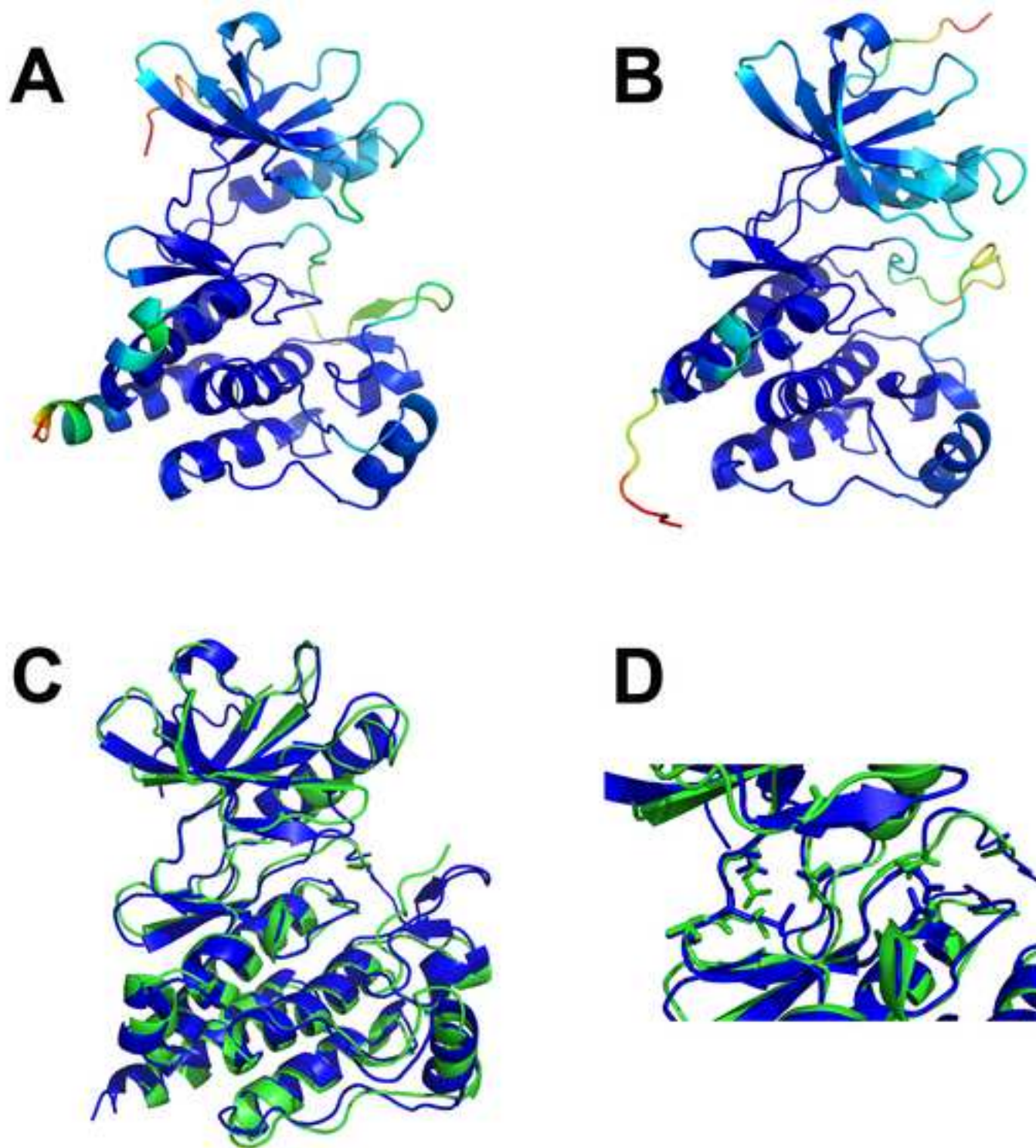


Figure 4
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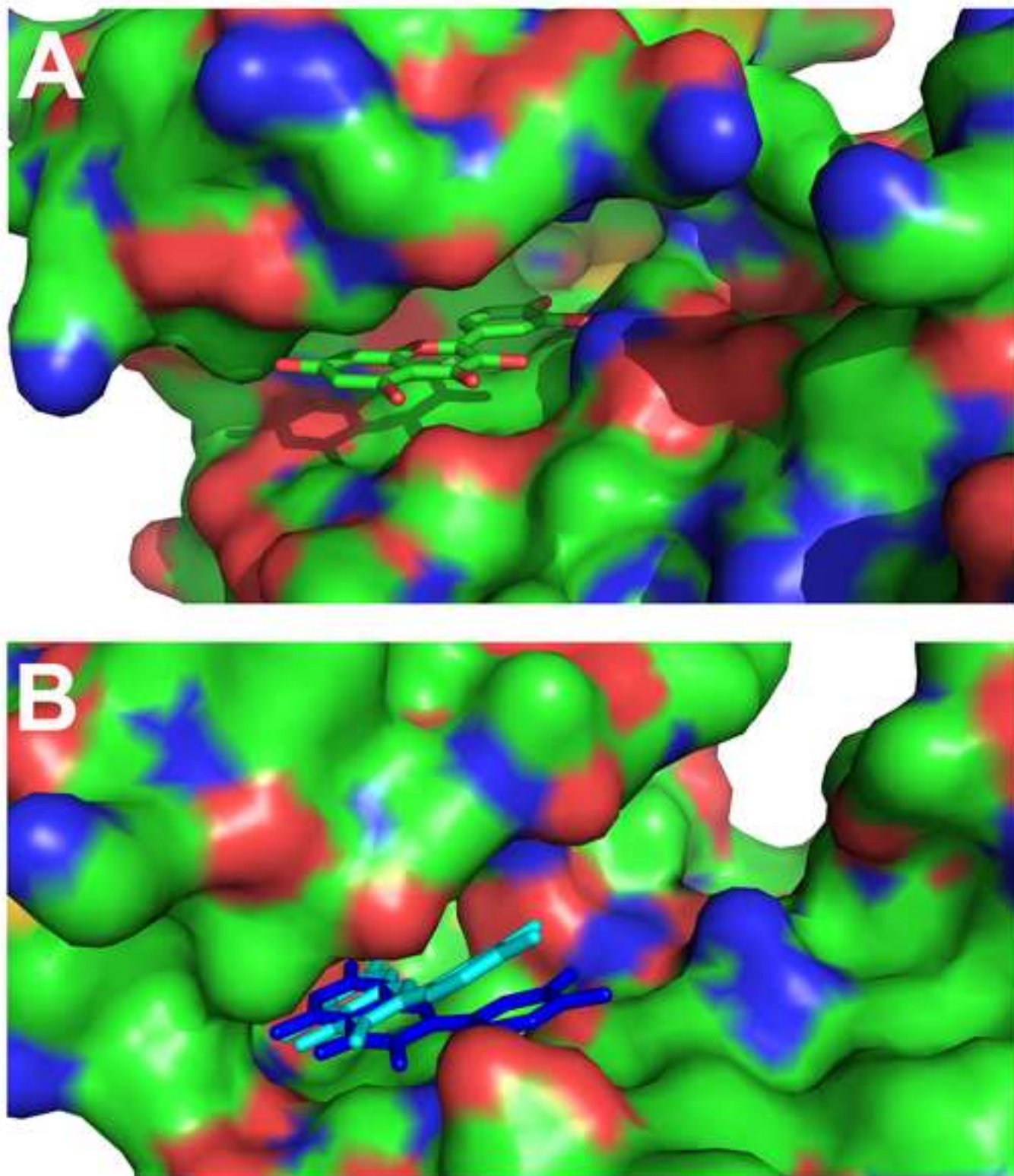


Figure 5
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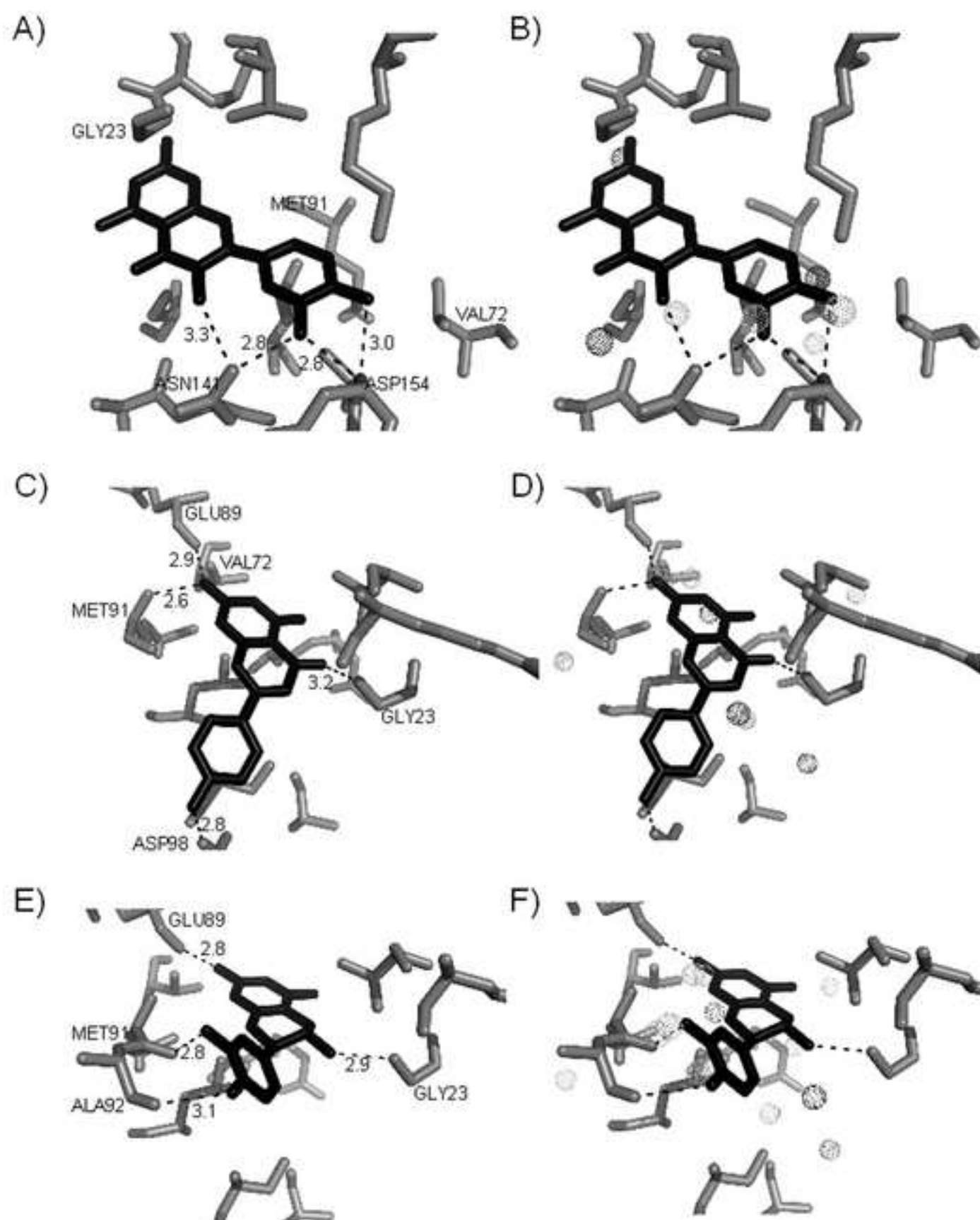
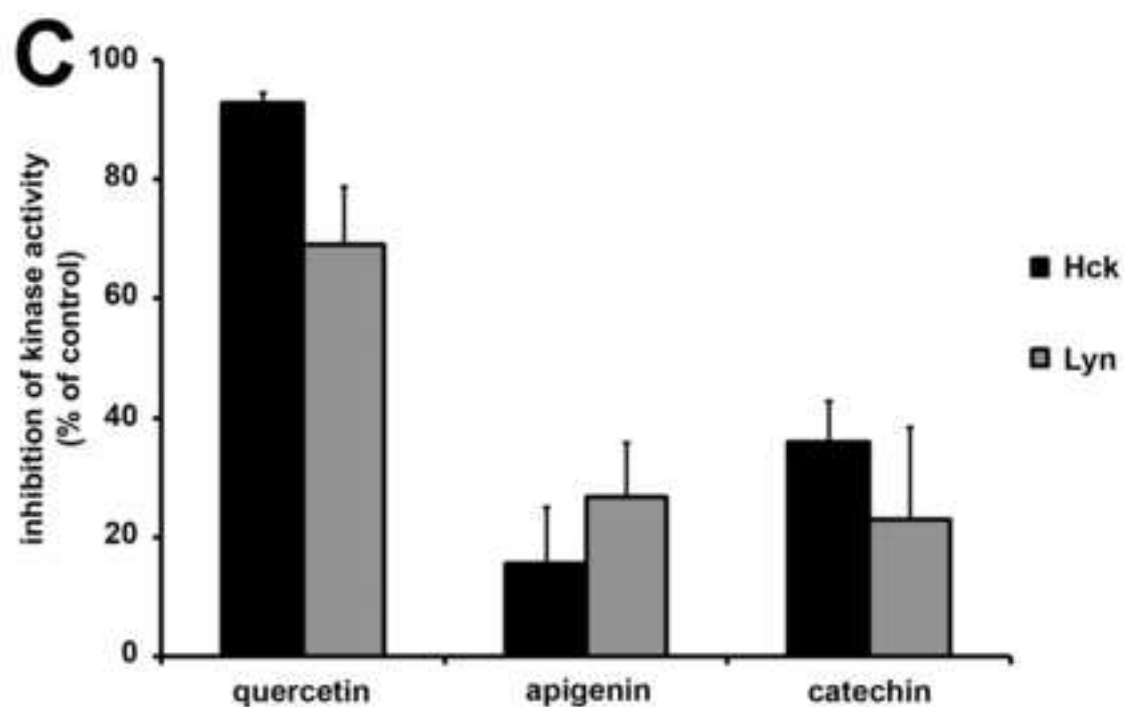
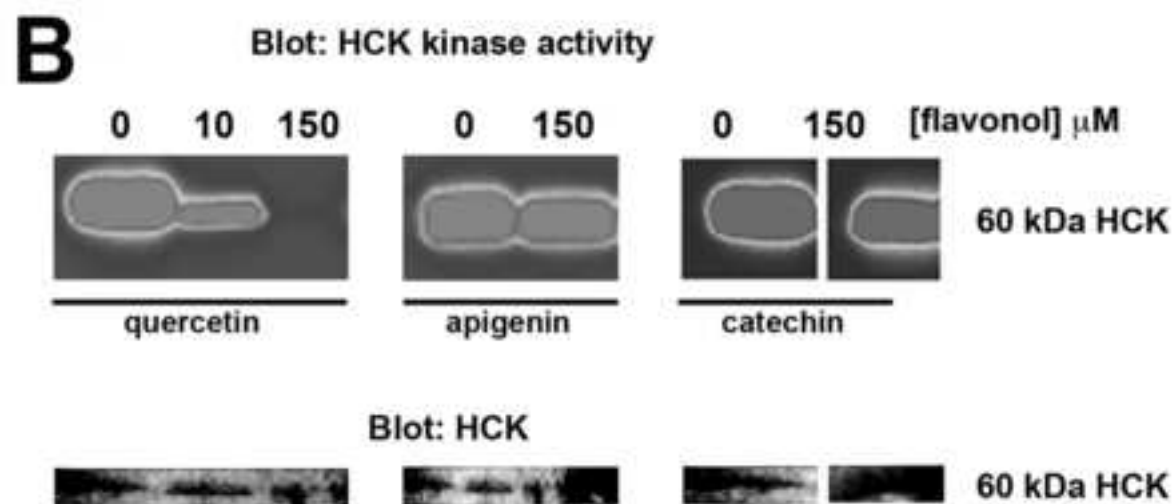
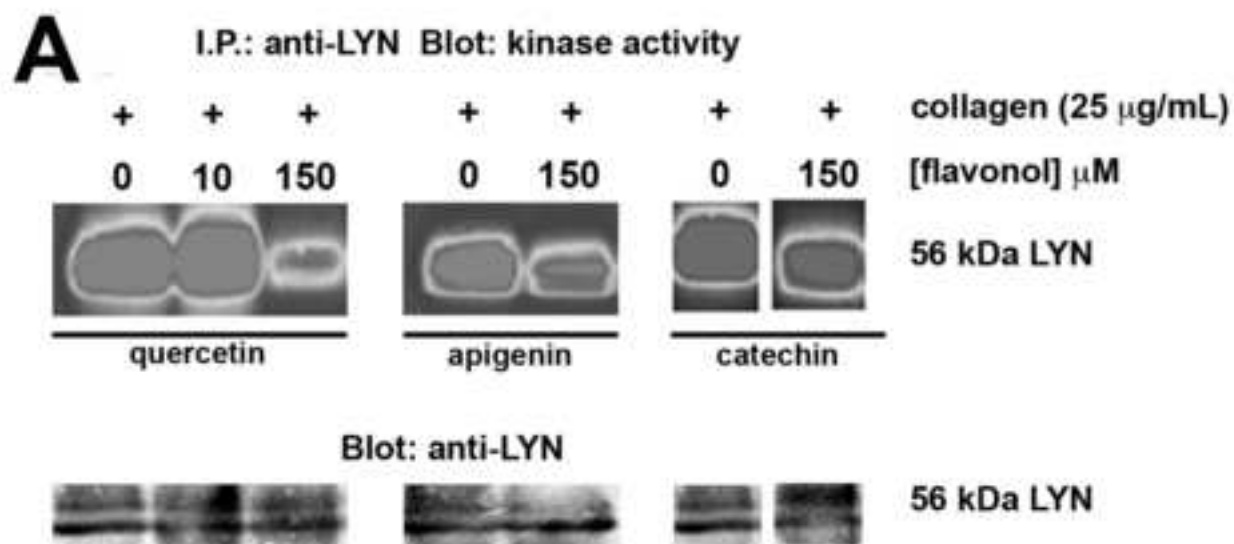


Figure 6
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Supplemental Figure 1

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Supplemental Figure 2

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